THE GENETICS OF SALMONELLA   RG-1445(c2)

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Joshua Lederberg
Associate Professor of Genetics
University of Wisconsin, Madison.

SUMMARY

Current work suggests that, under certain conditions, Salmonella cul-
tures form reduced cells which a) readily pass filters retaining the usual
bacteria; b) are more resistant to disinfection (heat, alcohol, chloroform);
and c) remain dormant in the absence of a stimulus from living cells. If
verified, these findings might require a reexamination of concepts of
bacteriological sterility.

This result was unexpectedly encountered in connection with work on
the mechanism of genetic recombination. Such a process, analogous to that
established in Escherichia coli K-12, seems to occur in some strains of
S. typhimurium. Whether there is a specific connection between the filtrable
agent and genetic recombination, as appears possible, is the subject of
current experiments.

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Recapitulation and Introduction

This research project on genetic aspects of the biology of Salmonella is directly related to previous and concurrent studies on genetic recombination in the related group of coliform bacteria. It will help to outline progress and aims of the Salmonella project if the results from Escherichia coli are briefly recapitulated.

About five years ago, E. L. Tatum and the undersigned (at Yale University), discovered that genetic recombination occurs among cells of E. coli strain K-12 (1,2). This was demonstrated by mixing cells from different nutritionally exacting or auxotrophic mutant cultures on a synthetic agar medium. By suppressing the auxotrophic parents, this medium selects for a small proportion of cells which have become prototrophic, i.e., able to form colonies in the absence of supplementary growth factors. With appropriately developed mutant stocks it was possible to rule out the possibility that these prototrophs arise from intrinsic instability of the mutant parents; they could arise only from the interaction of the distinct mutants: e.g., Ab with aB give rise to AB. This type of recombination process immediately suggested the possibility of a sexual process in this bacterium. This possibility has been reinforced by a number of experiments which showed the following:

1) Recombination was not confined to nutritional factors. Many other genetic differences introduced with the parents (e.g., fermentation characters, virus resistance, drug resistance) reassort in all possible combinations among the prototrophs.

2) Recombination occurs pairwise. In mixtures of three kinds of mutants, only those recombinants occur which could arise from pairwise exchanges, whereas uniquely tri-partite exchanges are not found.

3) In certain crosses, cultures ("heterozygous diploids") have been isolated which carry the genetic factors from both parents, which may later segregate during the further proliferation of the culture (3). Single cell studies showed conclusively that this segregation involves the separation of intra-cellular units, not entire single cells (4).

4) Numberous attempts to effect genetic exchanges by means of culture filtrates, cell extracts, or other preparations not containing normal viable cells of both parents have failed completely. This requirement for intact cells from both parents supports the concept that the fusion of ordinary vegetative bacterial cells is the basis of genic conjunction (as in many other microbes) and that no special gametic forms need be invoked. However, the actual fusion of cells has not been observed in this material, so that our conclusions on this detail of the "sexual" process are entirely inferential.

Until recently, evidence for gene recombination in bacteria was confined to strain K-12 of E. coli. Cavalli, working at Cambridge, England, has discovered a strain there which can be crossed with K-12 (5) and more recently a considerable number of strains have been isolated (about 3% of a series of 500 tested) which also show this phenomenon (6). Many of these new "crossable" strains are quite different from K-12, some being classifiable as paracolon or as coliform intermediates. This result makes it all the more necessary and hopeful to scrutinize other bacteria for similar genetic processes. Two objectives are preeminent: a) to provide the basis for genetic analysis of problems unique to other bacterial groups, e.g.- antigenic and pathogenic variation in Salmonella, and b) to find better material for the determination of the mechanisms, scope, and ecological role of recombination.

Experimental Results with Salmonella

Considerable time was spent during the first two years in collecting suitable cultures, developing techniques for producing biochemical mutations in Salmonella (7), and in perfecting the training of the research assistant assigned to this problem (Mr. Norton D. Zinder). After an interval during which mutants were induced in a diversity of types, including S. typhimurium, poona, madelia, "coli", and others, it was decided to concentrate on a coherent set of cultures of S. typhimurium.

Such a set was provided by Dr. S. Lilleeungen (Stockholm) who had worked out a procedure for bacteriophage typing of this species. He kindly placed at our disposal representative cultures of each of his 22 types of worldwide origin. In this way we could be fairly sure of covering a comprehensive sample of S. typhimurium cultures without unnecessarily reduplicating our work.

Auxotrophic mutants have been induced in 20 of these types. The present material has allowed crossing tests (like those in E. coli K-12) to be made in about half of the 200 possible combinations (inter- and intra-strain crosses); the mutants required to complete all of the possible combinations are being produced from day to day. It should be pointed out that, despite technical advances, the production and characterization of at least two double auxotrophic mutants in each of 20 strains represents a considerable multiplication of effort and has occupied the larger part of the time spent on this project to date.

Of the 99 combinations so far tested, 9 have more or less consistently given prototrophs on minimal agar medium, while the parents separately do not form colonies under these conditions. This is preliminary presumptive evidence for recombination in Salmonella typhimurium. Some of the combinations have given very low yields of prototrophs, a result which hinders the further study of the mechanisms of the apparent recombination. Our effort has been focussed on one particular combination which proved to be exceptionally fertile. Culture "A" is a mutant derived in two steps from Lilleeungen's type 2, "B" from type 22. "A" requires histidine and methionine "B" phenylalanine "plus" tyrosine, and tryptophane. Cultures of A or of B by themselves have never been found to produce prototrophs, even when very dense suspensions were plated on minimal agar. However, mixtures of A with

B have given yields of prototrophs of at least 10^{-5} of the parental inoculum, (considerably higher than has been found in E. coli). Since this combination gives the highest yield of presumably recombinant prototrophs, it was selected for further study. Two directions are being followed a) the genetic rules of recombination, and b) its biological mechanism.

With respect to a) certain peculiarities have been noted already. Attempts to induce fermentation mutants in A have been mostly unsuccessful; a number of mutants have been induced in B, and such stocks as "B" Gal- Xyl- (galactose-, xylose-negative) developed; in distinction to the Gal + Xyl + characteristic of the original A and B. "Gal" and "Xyl" are here used as unselected markers, i.e., the distribution of + and - qualities among prototrophs of A Gal+ Xyl+ x B Gal- Xyl- is followed. So far, the prototrophs have been almost all Gal- Xyl- like the "B" parent, and a like result has been obtained with other markers. However, a very small proportion of Gal+ Xyl- and Gal- Xyl+ have been formed. The disproportion of types might be due to genetic linkage, but more work will be needed to clear this up.

It has also been noticed that many fermentation mutants lose the "fertility" characteristic of the original culture. This probably represents an inherent instability in the capacity to react with other strains which must be given close attention in our survey.

The most unexpected results deal with the mechanism of genetic interaction, and have been purposely left to the last. In view of their rather heterodox character, they will have to be subjected to more than usual scrutiny, and tested in other laboratories before they can be entirely acceptable to any large body of workers, including ourselves.

These experiments began with one modelled after a report by B. D. Davis (8). A U-tube was constructed with an ultra-fine sintered Pyrex filter in the horizontal arm. The tube was sterilized and filled with broth. "A" was inoculated in one arm, "B" in the other. By alternating suction on the two sides, the medium was flushed from one compartment to the other until the cells had become so dense as to clog the filter. Several experiments in which one side only was inoculated with A or B confirmed the integrity of the filter, and the stability of the parent cultures.

The cells in each compartment were harvested and washed separately. Neither A nor B cells from control experiments gave any prototrophs on minimal agar. However, the B cells from the U-tubes in which the opposite arm contained A, repeatedly gave numberous prototrophs; the A cells did not. As in the experiments in which the cells were mixed directly, most of the prototrophs carried the unselected markers of B, but other types have also been noticed. Evidently, the interaction of A with B involves an "agent" produced by A which can pass a filter that retains the typical cells of A and B. The agent has been studied further in culture filtrates and other preparations. It is not produced (except to a very limited extent in aged cultures) by A or B cultures separately. Mixed cultures of A and B grown for several hours were sedimented and the supernatants passed through two Mandler filters, one medium, one extra fine, (the first of these usually

suffices for sterile filtration). 0.1 ml. of such a filtrate plated with 10^8 cells of B usually yielded ca. 100-200 prototrophs. The filtrates themselves were sterile by the usual criteria (no colonies on synthetic or complete agar medium; no turbidity in yeast extract broth).

Two filtrable factors are demonstrated by these experiments: 1) from B which stimulates A to form the agent 2) which reacts with B to form prototrophs. The first factor is probably a latent lysogenic bacteriophage secreted by B, as it can be propagated on A concomitantly with phage lysis. It can be replaced by sublethal concentrations of crystal violet; other deleterious treatments are being studied.

The agent is readily assayed by plating test samples with washed B cells on minimal agar. The number of prototrophs formed is proportional to the volume of a given filtrate tested, i.e., the assay is linear. The agent is more resistant than ordinary cells to inactivation by heat, chloroform, benzene, or alcohol, and is relatively unaffected by exposures which effectively sterilize the cells of B. It is apparently nondialyzable. It is precipitated by 60-70% ethanol or 60% saturated ammonium sulfate; the sediment redisperses readily in water. The agent has also been sedimented directly from filtrates by ultra-centrifugation in the Spinco centrifuge. These findings simplify the concentration and preparation of the agent.

We have not succeeded in extracting the agent from cells of B killed by heat, or subjected to autolysis under conditions proven harmless to the agent itself. This and later findings suggest that the agent is a biological product rather than an intracellular component of B.

The most obvious interpretations of the agents are:

1. as a "transforming agent" similar to those of pneumococci or Hemophilus influenzae.

2. as a minute cell product, a "gamete", or a form similar to the L-forms reported by several other authors (see 9).

The following results are especially tentative but incline to the latter hypothesis. Microscopic examination of active sediments shows barely visible granules and rods resembling ordinary bacteria except for their greatly diminished size. Probably more important, platings of apparently sterile filtrates with various kinds of cells, including Escherichia coli, have resulted in colonies with the same cultural characteristics and mutant markers as the original B. This suggests that the agent consists of reduced cells which are a) "filtrable" b) are more resistant to antiseptic treatments, and c) will remain dormant except in the presence of living bacteria. The possible implications of this tentative result for broad problems of antisepsis and "sterility" are obvious. In addition, it should be taken into account in reviewing the mechanisms of "transformations" reported for various bacteria. Whether they have a unique sexual or gametic function is prblematical. In preliminary experiments, however, the filtrates may have evoked prototrophs more readily and from a wider range of mutant cultures than did intact cells of B.

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